# Concentration and chemical stability of commercially available insulins – A high-resolution mass spectrometry study

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**Abstract**

Adequacy of insulin concentration in commercially available insulin formulations has recently been challenged. We therefore repeatedly evaluated insulin content and stability of 58 insulin vials containing five different insulin formulations (human insulin, standard/faster-acting insulin aspart, insulin lispro and insulin glargine) over a period of 85 days. High-resolution mass spectrometry was used to quantify intact monomeric insulin in glass vials and plastic pump cartridges exposed to three different temperatures (4, 22, 37°C), simulating real life conditions. In all cases, measured insulin concentration was in accordance with FDA and EMA requirements without evidence of chemical instability.

**Background**

To ensure optimal treatment and patient safety, rigorous quality control and assurance of therapeutics is of utmost importance. According to FDA requirements, each manufacturer has to guarantee a concentration of 95-105 IU/mL of intact insulin in U-100 formulations. Manufacturers recommend to store unopened vials at 2-8°C and keep used vials for up to 28 days whilst avoiding exposure to extreme temperatures and sunlight.

A recently published study by Carter and Heinemann examined 18 randomly purchased vials of NPH and regular insulin using liquid chromatography-mass spectrometry (LC-MS) and found mean concentrations of 40.2 IU/mL with levels ranging from 13.9 to 94.2 IU/mL[2]. The authors attributed the findings to inappropriate handling along the distribution chain. Their report raised concerns among people with diabetes and health care providers, and controversy among manufacturers, clinicians and biochemists. Follow-up studies using nuclear magnetic resonance (NMR) spectroscopy and high-pressure liquid chromatography (HPLC) demonstrated that insulin content was maintained along the supply chain, thereby complying with FDA and EMA requirements[3, 4].

Determination of insulin concentration is usually performed using HPLC, the FDA accepted standard method. Whereas HPLC is highly accurate, analysis times are usually more than 15 min and therefore limit throughput[5]. Alternatively, mass spectrometry (MS) is very well suited for peptide analysis[6] and there are numerous accounts of LC-MS being a highly sensitive and specific for detection of insulin and its analogues[7, 8]. In an attempt to challenge the findings of Carter and Heinemann, we set out to develop a high-throughput (1 min/sample) high-resolution MS method to quantify insulin content and stability in vials.

## Methods

*Collection of insulin formulations*

A total of 58 U-100-vials comprising insulin aspart (Novorapid®), faster-acting insulin aspart (Fiasp®), human insulin (Actrapid®), (all Novo Nordisk, Bagsvaerd, Denmark), insulin glargine (Lantus®; Sanofi-Aventis, Paris, France) and insulin lispro (Humalog®, Eli Lilly, Indianapolis, United States) were purchased from the hospital pharmacy (unopened; total of 31) or were obtained from wards of University Hospital Bern or patients (used; total of 27). All vials were stored according to the specifications of the manufacturer until measurement. DANA R pump cartridges (Diabecare, Seoul, South Korea) were filled with Fiasp®, Novorapid®, and Humalog® (unopened; in triplicate). Standards for human insulin, bovine insulin, lispro and glargine were obtained from Sigma-Aldrich (Buchs, Switzerland), aspart from LGC Standards (Teddington, UK). Hydrochloric acid and acetonitrile were purchased form Merck (Zug, Switzerland), formic acid from Thermo Fisher Scientific (Rheinach, Switzerland). All reagents were used in the highest obtainable quality.

To assess potential changes in insulin content and stability over time, formulations were sampled from vials and cartridges on days 1, 2, 3, 4, 5, 8, 9, 10, 11, 12, 15, 16, 17, 54 and 85 after receipt from the hospital pharmacy upon storage at either 4, 22 and 37°C in temperature-controlled environments with electronic temperature monitoring (either fridge or incubators).

*Determination of insulin content*

Mass spectrometric measurements were performed on a Q Exactive Orbitrap (Thermo Fisher Scientific, Switzerland) equipped with an electrospray ionisation source. Measurements were performed in the range m/z 800-1500 at R=70000 in positive ion mode. Samples were injected using the autosampler and pump modules of a Vanquish system (Thermo Fisher Scientific, Switzerland).

As we only analyzed drug formulations of minor complexity and high concentrations in this study, we decided to forego the HPLC step and directly analyze the vial contents by MS for increased throughput.

Such flow injection setups, which omit a pre-separation of analytes, are widely employed in metabolomics and lipidomics[9, 10].

Reference compounds were dissolved in 0.01 molar hydrochloric acid and diluted using 33% acetonitrile in water to desired concentrations. External and internal calibration was performed using a 6-point calibration curve between 0.2 and 54 umol/L. Internal standard was added to all insulin therapeutics, followed by 50-fold dilution using 33% acetonitrile to generate monomeric insulin, resulting in a theoretical insulin concentration of 12 umol/L in polypropylene 96 well plates (Eppendorf, Switzerland). Samples were then stored in the autosampler at 10°C and measured within 1 h after dilution. All samples and calibrants contained bovine insulin at a final concentration of 2 umol/L as an internal standard.

*Statistical analyses*

Change in insulin content was determined using linear regression. A significant decline in concentration was considered a slope of the regression line significantly different from zero, evidenced by $p<0.05$. Statistical analysis was performed using R (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (GraphPad Software, La Jolla, USA).

**Results**

For all compounds in the present analysis the quantification of monomeric insulin using our mass spectrometric method showed linear response over the entire calibration range ($R^2$ ranging from 0.9948 to 0.9988), accuracy of between 96% and 104%, and precision between 2.4 to 3.8% (Supplemental Table S1).

The measured insulin content of the 58 vials comprising five different drug formulations fell in the required concentration range of within 5% of the nominal value. No systematic difference between unopened vials (obtained from the pharmacy) and already used vials (obtained from hospital wards and patients) was observed. Likewise, the time-course experiment during which the insulin content of the different formulations stored at different temperatures (4, 22 or 37°C) was investigated yielded results according to specification. An example of such a longitudinal assessment at different temperatures is shown in Figure S1 for Humalog®. The statistical evaluation of the long-term content assessment for all compounds stored in vials is summarized in Table 1.

Screening for degradation products throughout the study turned out negative. An example of a theoretical degradation product resulting from deamidation is illustrated in Figure S2.

**Table 1.** Concentration assessment of insulin formulations stored in original glass vials (Novorapid®, Fiasp ®, Humalog®, Actrapid® and Lantus®) and cartridges (Novorapid®, Fiasp®, Humalog®) at three different temperatures. Mean is average concentration over all measurements (15 time points over 86 days). CV = coefficient of variations over all measurements. p = p-value of the regression line (concentration versus time). A p-value >0.05 indicates no change in concentration over time.

| | **Novorapid® in vial** | | | **Fiasp® in vial** | | | **Humalog® in vial** | | | **Actrapid® in vial** | | | **Lantus® in vial** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (U/mL) | CV % | p | Mean (U/mL) | CV % | p | Mean (U/mL) | CV % | p | Mean (U/mL) | CV % | p | Mean (U/mL) | CV % | p |
| 37°C | 98.5 | 5.4 | 0.44 | 99.1 | 3.5 | 0.96 | 99.5 | 6.7 | 0.81 | 98.7 | 4.6 | 0.45 | 95.4 | 3.7 | 0.32 |
| 22°C | 99.3 | 3.9 | 0.67 | 97.7 | 4.8 | 0.88 | 101.6 | 5.2 | 0.91 | 99.4 | 5 | 0.95 | 97.7 | 5.5 | 0.42 |
| 4°C | 99.7 | 3.2 | 0.74 | 100.7 | 4 | 0.87 | 102.9 | 3.9 | 0.59 | 98.6 | 5.5 | 0.55 | 98.4 | 4.6 | 0.43 |
| | | | | | | | | | | | | | | | |
| | **Novorapid® in cartridge** | | | **Fiasp® in cartridge** | | | **Humalog® in cartridge** | | | | | | | | |
| | Mean (U/mL) | CV % | p | Mean (U/mL) | CV % | p | Mean (U/mL) | CV % | p | | | | | | |
| 37°C | 100.3 | 3.9 | 0.87 | 102.4 | 5.9 | 0.68 | 101.5 | 3.7 | 0.82 | | | | | | |
| 22°C | 101.4 | 3.4 | 0.59 | 100.5 | 5.9 | 0.75 | 101.4 | 3.2 | 0.64 | | | | | | |
| 4°C | 100.8 | 4.2 | 0.68 | 102.5 | 7.1 | 0.68 | 100.6 | 4.7 | 0.79 | | | | | | |

**Discussion**

The present study repeatedly determined the insulin content and chemical stability of 58 insulin vials containing five different analogues over a period of almost three months. During the study period, glass vials and plastic cartridges were exposed to three different temperatures (4, 22, 37°C), simulating real life conditions. Despite stressed conditions (storage duration and temperature exposure beyond manufacturer's recommendations) the measured insulin content was in accordance with FDA and EMA requirements and we did not find any evidence of chemical instability.

Despite the use of a similar analytical approach, our findings are inconsistent with the results obtained by Carter and Heinemann[2]. Conversely, our data is in line with results by Moses et al who reported insulin concentrations within the specified range for hundreds of different vials using HPLC which is currently the recognized FDA standard[4]. Even though our MS assay exhibits similar accuracy when compared to HPLC (standard deviations ranging from 2.4 to 6.7% for the HPLC method and 3.9-5.1% for the MS method as evidenced in Figure S1, supplementary information), the sample-to-sample time of the MS assay is 1 min compared to at least 15 min for HPLC. Consequently, the MS assay may have potential for large-scale application.

Our results further support the suggestion brought forward by others[3, 4] that there were potential issues with the analytical method employed by Heinemann and Carter. We can only speculate on the reasons for the discrepancies, which could include steps during sample dilution (precluded in our case by the use of an internal standard) or the use of a low-resolution mass spectrometer. Our results also conform with recent findings obtained by NMR spectroscopy[3]. When compared with NMR and HPLC, MS confers the benefit of offering automation and high-throughput analysis. Yet, all discussed analytical techniques (HPLC, MS, NMR) suffer from the same limitation: biological activity, which could potentially be impacted through changes in the tertiary protein structure or protein aggregation upon storage, cannot be assessed. Determination of actual biologic insulin activity requires use of specialized

assays to monitor insulin receptor activity and binding or the evaluation of glycemic effects in animal models or humans using the hyperinsulinemic euglycaemic clamp method [11].

**Conclusions**

In summary, the present high-resolution MS study demonstrated that insulin content in vials, even when exposed to suboptimal temperature conditions, remained within the acceptable limits specified by FDA and EMA without evidence of degradation. From a technical point of view, we established flow-injection high-resolution MS as an attractive tool for high-throughput determination of insulin concentrations. The method allows accurate measurement of compound concentrations in 1 min, is capable of directly detecting degradation such as deamidation or oxidation and therefore represents an alternative to HPLC-based methods.

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**Duality of Interest**

F.B., C.S., B.V., L.B., and M.G. declare no competing financial interest.

**Author Contributions**

LB and MG designed the study. FB and MG performed the experiments, collected, processed and analyzed the data. LB and MG wrote the manuscript. BV and CS critically reviewed the manuscript. LB and MG are the guarantors of this work, as such, had full access to all the data in the study, and take the responsibility for the integrity of the data and the accuracy of the data analysis.

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